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ECDYSONE METABOLISM AND THE INTERRUPTION OF PROECDYSIS IN THE LAND CRAB, *GECARCINUS LATERALIS*[1]

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In the brachyuran crabs, there is a complex interaction between limb regeneration and the intermolt cycle. The majority of limb regeneration occurs during the premolt period of the intermolt cycle (proecdysis; stage D) during which the animal undergoes preparation for ecdysis. However, regeneration can, in turn, influence the progress of proecdysis: autotomy of limbs or partially regenerated limbs before a critical stage in proecdysis (stage D_1) results in a temporary suspension of proecdysis while a new round of regeneration occurs at the sites of the missing limbs. During this suspension in stage D_0, not only is there a cessation of the growth of partial regenerates remaining on the crab (Holland and Skinner, 1976), but gastrolith deposition and the cytological activation of the epidermis are also delayed. This suspension of proecdysis is associated with a rapid decline of serum ecdysteroid titers from $\sim$ 70 to $\sim$ 15 ng/ml within a day of the autotomy; ecdysteroid titers remain low for two weeks while the recently autotomized limbs undergo basal regeneration. Titers then re-initiate their normal pattern of proecdysial increases; events which had been temporarily suspended resume development in association with the increased hormone titer so that the animal undergoes a normal, but delayed, ecdysis and releases a full compliment of regenerated limbs (McCarthy and Skinner, 1977b).

This interaction between regeneration and the molt cycle suggests that changes in the titer of 20-hydroxyecdysone, the biologically active ecdysteroid, regulate the progress of proecdysial events; and that the accepted paradigm of molt regulation (Passano, 1960), is incomplete, since these same interactions occur even in animals deprived of eyestalks, the source of the molt-inhibitory hormone (McCarthy and Skinner, 1977b).

In this paper, the metabolic fate of ecdysone [α-ecdysone, the secretory product of the Y-organ (Chang and O'Connor, 1977)] is examined in animals in late stage D_0 either with or without the simultaneous autotomy of four partially regenerated limbs. The purposes of this study are: to support and extend our earlier studies on ecdysteroid metabolism in crabs in the intermolt (stage C_4; McCarthy and Skinner, 1979) and mid-to-late premolt (stages D_1 to D_2 and stage D_3; McCarthy, in preparation) stages of the intermolt cycle; to examine the biochemical mechanisms by which the rapid decline in ecdysteroid titers is achieved following limb autotomy in stage D_0; and to provide additional information on the nature of the regulatory mechanisms responsible for the interruption and re-initiation of proecdysis.

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Material and Methods

Animals

Specimens of the land crab, *Gecarcinus lateralis,* ranging in weight from 30 to 60 g were obtained from the Bermuda Biological Station and were maintained in the dark and fed weekly. The animals were induced to enter proecdysis precociously by causing the animals to autotomize 8 walkings legs (Skinner and Graham, 1970, 1972). Premolt animals in late stage D_0 were used. They were determined to be in late stage D_0 by the following criteria: limb regenerates were at an R-value between 14 and 17 (Bliss, 1956; Skinner, 1962); serum ecdysteroid titers [estimated by radioimmunoassay (RIA); Horn, Wilkie, Sage and O'Connor, 1976] were between 60 and 90 ng/ml (McCarthy and Skinner, 1977a, b); and the epidermis was still firmly attached to the cuticle of the branchiostegite region. Crabs met these criteria approximately 30 days after molting was induced by multiple limb autotomy.

To examine the mechanisms by which endogenous ecdysteroid titers are rapidly decreased when limb regenerates are re-autotomized in stage D_0, another set of metabolic studies was conducted on crabs meeting the above criteria for stage D_0; however, in these animals, four of the partially regenerated limbs were re-autotomized immediately before the [^{3}H]ecdysone was injected (at $t = 0$ hr in Figs. 1B, 3, 4B). This group of animals are designated as "LBA crabs" (limb bud autotomized). Crabs in stage D_0 which did not have limbs re-autotomized are sometimes referred to as "normal crabs in stage D_0" to distinguish them from the LBA crabs.

Injection and extraction of ecdysones

A small mass of high specific activity α-[23,24 ^{3}H]ecdysone ($\sim$ 55 Ci/millimole) was diluted in perfusion fluid (Skinner, Marsh and Cook, 1965) and a total volume of 2–3 μl/g live wt. (0.3–0.5 ng ecdysone/g live wt) was injected into the hemolymph sinus at the base of a chela. The [^{3}H]ecdysone, which was a gift of Dr. D. S. King (Zoecon Corp., Palo Alto, CA), was chromatographically purified prior to use (see below). At 1, 4, 9 or 24 hr after injection crabs were chilled on ice and dissected. Samples of hemolymph (from the chela sinus) and epidermis (from the branchiostegite region) were removed as were midgut gland (including the midgut) and the hindgut. These tissues were rinsed in saline and they, as well as the remainder of the crab, referred to as "carcass," were frozen in liquid nitrogen and held at $-60°$ C.

For each time point tissues from 3 to 4 crabs were pooled and extracted 3 times in hot methanol. The pooled extracts were partially purified as previously described (McCarthy and Skinner, 1979). This crude purification procedure permitted recovery of 65 to 75% of the total radioactivity.

Chromatographic separation of ecdysteroids

Ecdysone metabolites in the partially purified extract were separated by high pressure reverse phase liquid chromatography (HPLC) on a 3.9×60-cm μBondapak C-18 column (Waters Assoc.) eluted with a linear gradient of 20 to 100% methanol in water over 165 min at a flow rate of 0.5 ml/min. A Model 6000 solvent delivery system with a Model U6K universal injector and a Model 660 solvent programmer (Waters Assoc.) were used for the analyses. The relative

TABLE I

Ecdysone and its metabolites in normal crabs in stage D_0. *Dashes indicate that the compound was not detected; "NR" is the very polar compound which was not retained by the column even at 20% methanol.*

Tissue	Hr after injection	Component (% radioactivity)						
		NR	Polar conjugate of			Free ecdysteroids		
			20,26-OH	β	α	20,26-OH	β	α
Serum	1	—	—	—	—	—	58	42
	4	—	—	—	2	13	81	4
	9	—	—	—	—	16	82	2
	24	—	—	—	—	14	85	1
Epidermis	1	—	—	1	—	1	50	48
	4	—	—	5	—	5	83	7
	9	—	1	10	—	17	69	3
	24	—	6	—	—	13	80	1
Carcass	1	—	3	—	9	11	29	48
	4	—	—	—	5	6	65	24
	9	—	—	—	—	14	72	16
	24	—	—	—	—	15	83	2
Midgut gland	1	—	—	7	72	—	4	17
	4	—	—	20	43	—	15	22
	9	—	—	30	39	3	12	16
	24	—	4	15	15	13	18	15
Hindgut	1	—	—	—	—	—	42	58
	4	—	—	10	10	5	74	11
	9	—	—	2	2	16	77	5
	24	15	—	—	—	13	72	—
Total crab	1	—	1	11	11	6	38	44
	4	—	—	9	9	7	64	18
	9	—	—	6	6	13	64	12
	24	—	1	3	3	15	70	4

contributions of the various metabolites were determined by liquid scintillation counting of 1-ml fractions of the eluent. Details of these procedures have been described (McCarthy and Skinner, 1979).

Rate coefficients

The rates of conversion, turnover and elimination of ecdysone metabolites are expressed as rate coefficients, k, estimated from the slope of the semilog disappearance curve (*e.g.*, Fig. 1). In cases where the semilog plot was clearly non-linear the rate constants for the fastest and slowest components were estimated by graphical analysis (Atkins, 1969). The data in Figures 2 and 3 are not presented as semilog plots; data were replotted for graphical analysis. The rate coefficients are expressed in inverse time and, in most cases observed in this study, varied from 0.002/hr ($t_{\frac{1}{2}}$ = 350 hr) to 0.10/hr ($t_{\frac{1}{2}}$ = 5.6 hr).

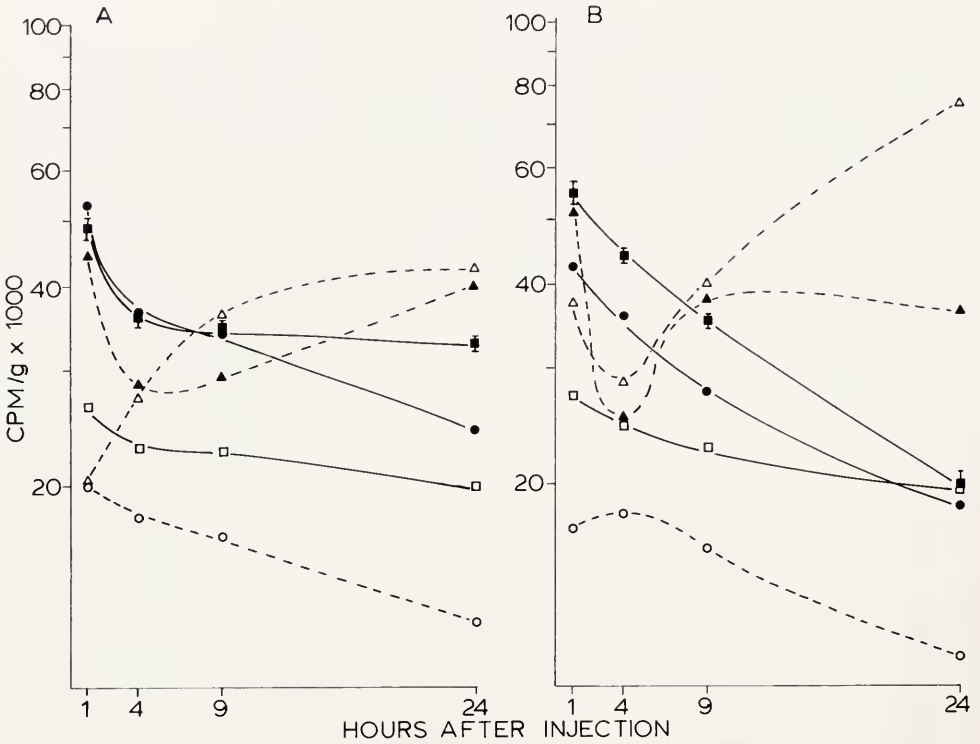

FIGURE 1. Concentration of total radiolabeled ecdysone and its metabolites following injection of [^{3}H]ecdysone in (A) normal crabs in stage D_0 and (B) LBA crabs in stage D_0 (with partially regenerated limbs autotomized at t = 0 hr). The pattern of change is indicated for serum (closed boxes), epidermis (closed circles), hindgut (closed triangles), total crab (open boxes), midgut gland (open triangles), and carcass (open circles). As an estimate of the variability within experimental groups, the radioactivity in the serum of individual crabs was determined and results are plotted as the mean ± 1 SEM.

RESULTS

Metabolites of ecdysone

[^{3}H]Ecdysone was very rapidly converted by tissues of crabs in stage D_0 to a total of five metabolites, two of which co-chromatographed with authentic 20-hydroxyecdysone and 20,26-dihydroxyecdysone (see Fig. 1 in McCarthy and Skinner, 1979). Three radiolabeled polar components were also observed. These components have been identified in intermolt crabs as polar conjugates, probably glycosides, of the three free ecdysteroids (McCarthy and Skinner, 1979). In the hindgut, a sixth radiolabeled metabolite was detected which was very polar and eluted from the reverse phase column in the void volume. A component with similar chromatographic properties was detected in the urine and feces of intermolt crabs (McCarthy and Skinner, 1979); this unidentified metabolite probably represents an excretion product of ecdysteroids.

Metabolic fate of ecdysone in various tissues

The change in the total radioactivity (Figs. 1A, B) and the relative amounts of the several ecdysone metabolites (Tables I and II) were determined in several

TABLE II

Ecdysone and its metabolites in LBA crabs. Symbols are as in Table I.

Tissue	Hr after injection	Component (% radioactivity)						
		NR	Polar conjugate of			Free ecdysteroids		
			20,26-OH	β	α	20,26-OH	β	α
Serum	1	—	—	—	—	1	56	43
	4	—	—	—	2	13	79	6
	9	—	—	—	—	19	78	3
	24	—	—	—	—	17	81	2
Epidermis	1	—	—	—	—	—	57	43
	4	—	—	—	6	9	73	12
	9	—	—	11	4	21	58	6
	24	—	—	11	—	15	72	2
Carcass	1	—	—	—	—	5	44	51
	4	—	—	—	—	3	65	32
	9	—	—	—	4	16	66	14
	24	—	—	—	—	24	76	—
Midgut gland	1	—	—	12	85	—	—	3
	4	—	1	26	62	—	—	11
	9	—	—	31	47	4	5	13
	24	—	4	43	21	8	14	10
Hindgut	1	22	—	—	—	—	42	36
	4	15	4	—	9	3	57	11
	9	28	6	—	7	14	42	3
	24	5	9	—	6	16	60	3
Total crab	1	—	—	2	12	2	43	41
	4	—	—	3	8	6	63	20
	9	—	—	5	11	15	59	10
	24	—	1	17	8	16	53	5

tissues at various times after injection of [^{3}H]ecdysone. The radioactivity present as each metabolite (cpm/g tissue) was also calculated (Figs. 2, 3).

Serum. The rate of loss of total radioactivity from the serum is rapidly accelerated by the autotomy of partially regenerated limbs. In crabs in stage D_0, radioactivity is rapidly lost for the first 4 hr ($k = 0.10$/hr), but the loss slows greatly during the remaining 20 hr of the experimental period ($k = 0.004$/hr). In crabs whose limb regenerates were re-autotomized at the time of injection, the rate of loss of radioactivity is rapid throughout the experimental period ($k = 0.04$/hr) (Fig. 1).

20-Hydroxyecdysone is the primary (> 80%) metabolite of ecdysone in both sets of crabs; however, the rate of loss of 20-hydroxyecdysone undergoes a twenty-fold increase from $k = 0.002$/hr for normal crabs in stage D_0 to $k = 0.04$/hr within 4 hr after LBA (Figs. 2A, 3A). The rate of loss of [^{3}H]20-hydroxyecdysone following LBA is approximately the same as the rate of decrease of serum ecdysteroid titers following LBA ($k = 0.046$/hr; McCarthy and Skinner, 1977b).

Epidermis. The loss of radioactivity from epidermis of crabs in stage D_0 has two kinetic components: a rapid loss initially ($k = 0.10$/hr), followed by a much

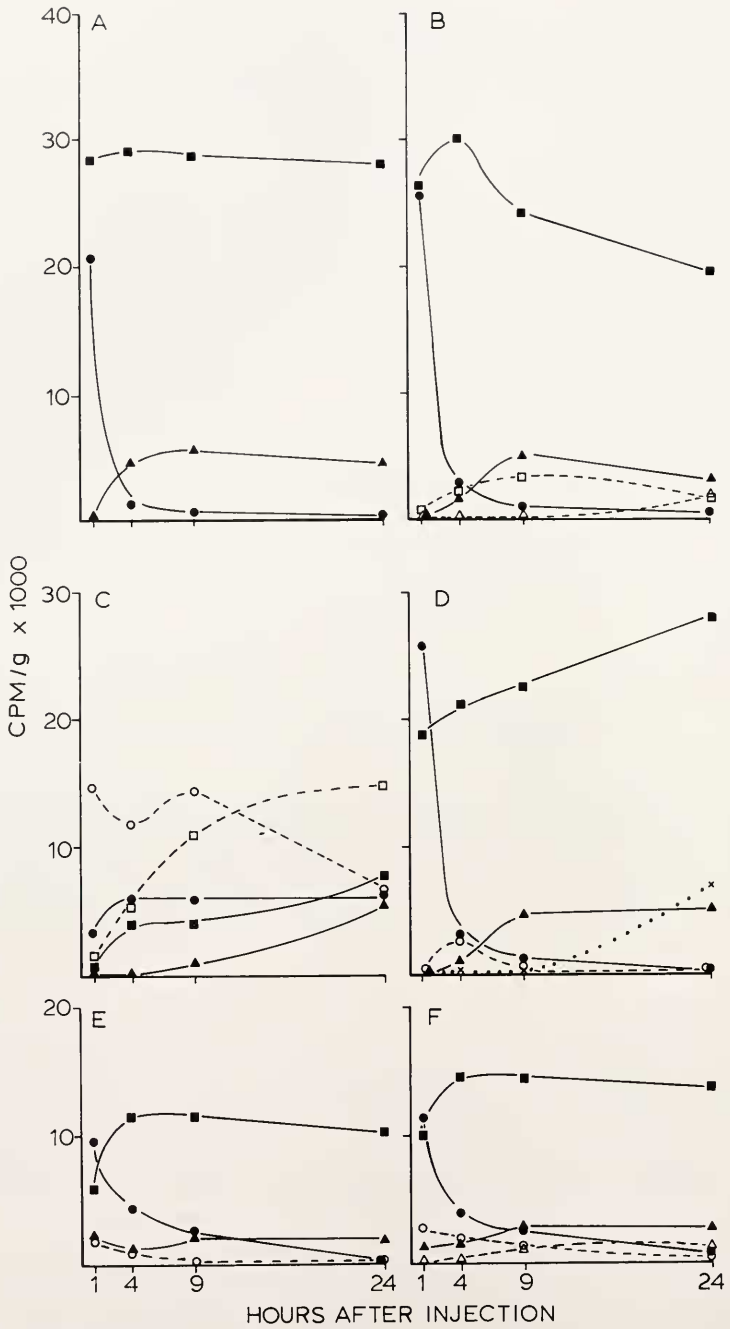

FIGURE 2. Concentration of [^{3}H]ecdysone and its metabolites in normal crabs in stage D_0. Free ecdysteroids are indicated by filled symbols and solid lines; conjugates of each ecdysteroid are indicated by open symbols and dotted lines: ecdysone (closed circles) and its conjugate (open circles); 20-hydroxyecdysone (closed boxes) and its conjugate (open boxes); 20,26-dihydroxyecdysone (closed triangles) and its conjugate (open triangles). The very polar metabolite which is not retained on the column even at 20% methanol is indicated by the crosses with dotted line. (A) serum; (B) epidermis; (C) midgut gland; (D) hindgut; (E) carcass; and (F) the total crab.

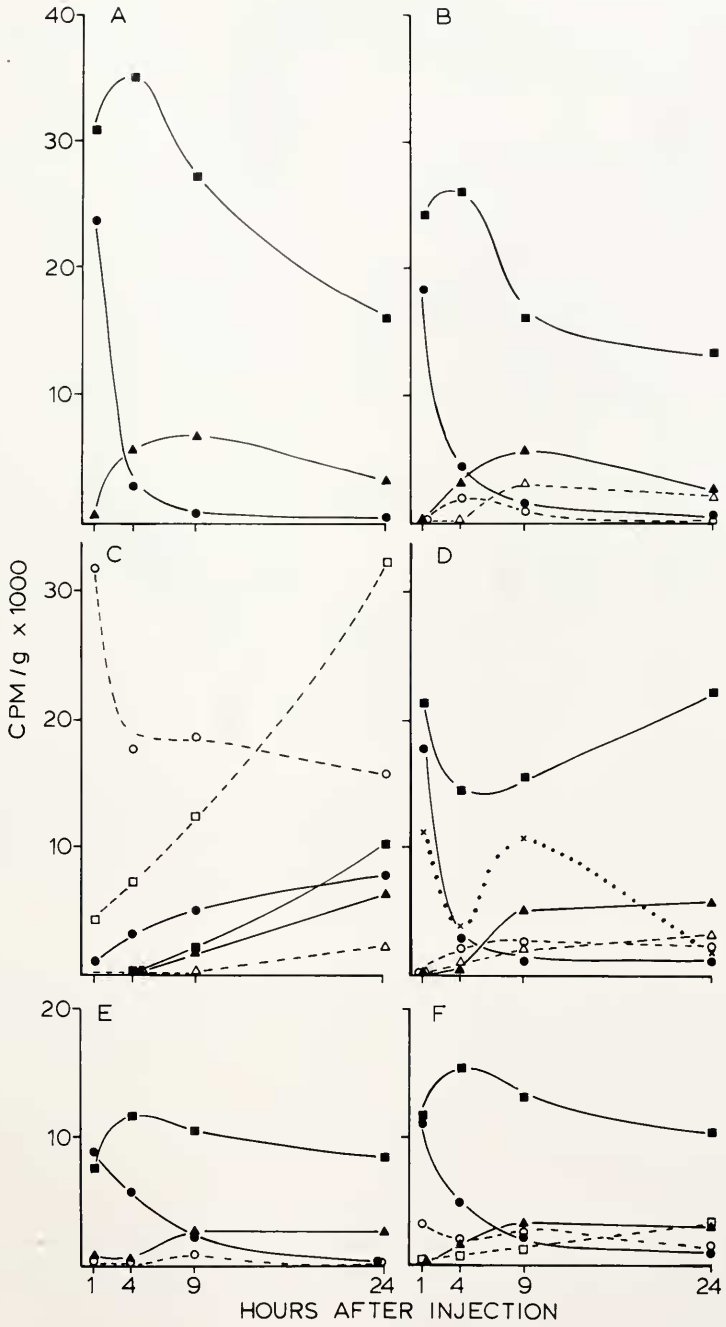

FIGURE 3. Concentration of ecdysone and its metabolites in LBA crabs. Legend same as in Figure 2.

slower loss during the remaining 20 hr ($k = 0.02$/hr). Following LBA, total radioactivity is lost at a relatively constant rate over the entire 24-hr period ($k = 0.04$/hr) (Fig. 1).

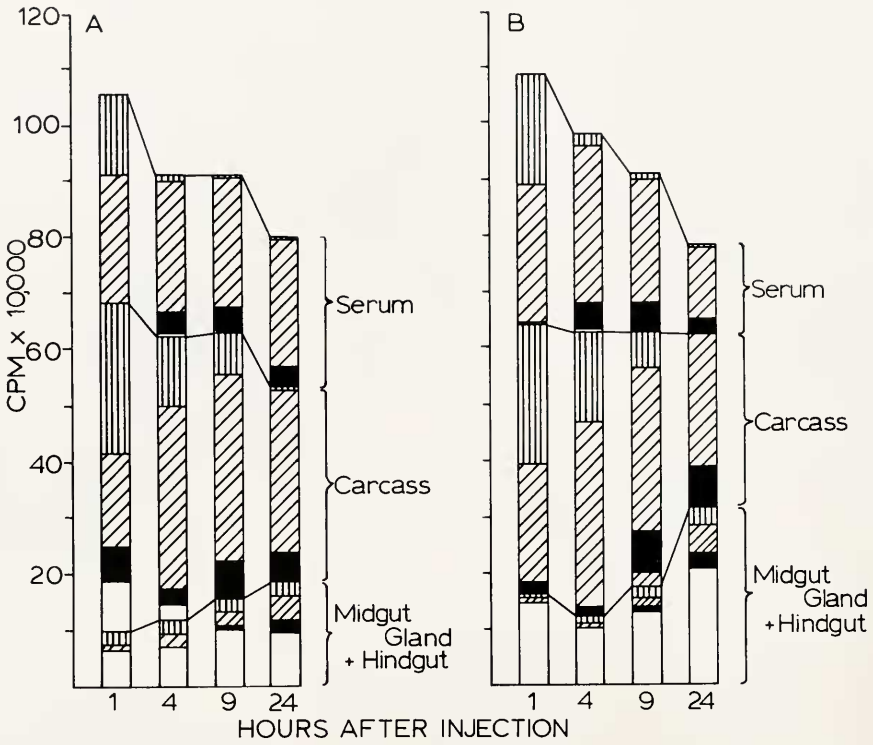

FIGURE 4. Pattern of change of radiolabeled metabolites in (A) normal crabs in stage D_0 and (B) LBA crabs. Changes in the total radioactivity in serum, carcass and midgut gland-hindgut over a 24-hr period are indicated by the diagonal lines between the bars for each time point. The amount of the various metabolites in each tissue at each time point is indicated: ecdysone (bars with vertical lines); 20-hydroxyecdysone (bars with diagonal lines); 20,26-dihydroxyecdysone (filled bars); total polar conjugates (empty bars).

20-Hydroxyecdysone is the primary metabolite of ecdysone; however, beginning at 4 hr, further hydroxylation to 20,36-dihydroxyecdysone becomes significant. The rate of loss of 20-hydroxyecdysone is slightly accelerated following LBA from $k = 0.02$/hr to 0.03/hr (Figs. 2B, 3B).

Carcass. The rate of loss of total radioactivity from the carcass is relatively unaffected by the re-autotomy of partial regenerates; the rate coefficients are $k = 0.020$/hr and 0.025/hr for normal crabs in stage D_0 and following LBA, respectively (Fig. 1).

20-Hydroxyecdysone and 20,26-dihyroxyecdysone are the primary metabolites observed in the carcass; the relative proportions of these metabolites increase steadily over the experimental period. The rate of loss of 20-hydroxyecdysone from the carcass more than doubles from $k = 0.006$/hr for normal crabs in stage D_0 to $k = 0.014$/hr within a few hours after re-autotomy (Figs. 2E, 3E).

Midgut gland. The major metabolites present in the mid-gut gland are polar conjugates of the ecdysteroids. The conjugate of ecdysone is the most abundant metabolite shortly after injection; however, as the concentration of this metabolite decreases, the amount of the conjugate of 20-hydroxyecdysone increases rapidly. In crabs in stage D_0, the amount of polar conjugates levels off toward the end

of the experimental period, but following LBA, the concentration (cpm/g) of polar conjugates continues to increase so that at 24 hr, more than twice as much of these polar inactivation products are present in crabs from which limb regenerates were re-autotomized at the time of injection compared to normal crabs in stage D_0 (Figs. 2C, 3C). The elevated amount of these polar products is probably related to an increased rate of loss of ecdysteroids following re-autotomy.

Hindgut. 20-Hydroxyecdysone is the primary metabolite detected in the hindgut and its concentration increases fairly rapidly, especially in the latter portion of the experimental period. The hindgut also contained a metabolite not observed in any other tissue. This very polar metabolite was observed in fairly low amounts at the end of the experimental period in crabs in stage D_0, but appeared earlier and in larger amounts following LBA (Figs. 2D, 3D). Since this metabolite was previously detected only in feces of intermolt crabs (McCarthy and Skinner, 1979), the feces contained in the hindgut of the crabs of stage D_0 may be the source of this metabolite.

Metabolic fate of ecdysone in the whole crab

In crabs in stage D_0, the overall pattern of ecdysone metabolism is characterized by: metabolism of ecdysone primarily to 20-hydroxyecdysone, with further hydroxylation to 20,26-dihydroxyecdysone occurring to a limited extent; and catabolism of free ecdysteroids to polar conjugates.

The rate of loss of 20-hydroxyecdysone from crabs in stage D_0 is very slow ($k = 0.002/hr$); however, the rate of loss undergoes a tenfold acceleration within hours after LBA ($k = 0.02/hr$; Figs. 2F, 3F). The increased rate of loss of 20-hydroxyecdysone from LBA crabs is associated with an increase in the relative proportion and total amount of the polar conjugate of 20-hydroxyecdysone (Fig. 4) accumulated in the midgut gland, suggesting that the enhanced elimination of the active hormone is accomplished through an increased rate of catabolism to polar inactivation products.

Discussion

Metabolism of ecdysone in land crabs appears to procede primarily through: hydroxylation of the sidechain at C-20, then C-26 to form 20-hydroxyecdysone (50–80%) and 20,26-dihydroxyecdysone (5–20%), respectively; and conjugation of the free ecdysteroids to form glycosides. These basic pathways were observed in animals in the intermolt stage (stage C_4; McCarthy and Skinner, 1979), early premolt (stage D_0), as well as the later stages of proecdysis (stages D_1–D_3, McCarthy, in preparation). The metabolic fate of ecdysteroids does change at the various stages of the intermolt cycle with respect to: the rate of elimination or turnover of 20-hydroxyecdysone; and the amount of catabolism of free ecdysteroids to their polar conjugates.

As the animal enters into proecdysis the rate of turnover of 20-hydroxyecdysone decreases about three-fold and the amount of further metabolism to 20,26-dihydroxyecdysone and catabolism to polar conjugates also decrease. This inverse relationship between the rate of catabolism and excretion of ecdysteroids and the endogenous ecdysteroid titer during the period of hormone increase has also been observed in several insects (Koolman, Hoffmann and Dreyer, 1975; Hikino, Ohizumi and Takemoto, 1975; Feyereisen, Lagueux and Hoffmann, 1976; Weinheimer and Romer, 1977) and may be a regulatory mechanism common to arthropods.

The molt-related changes in the rates of catabolism and elimination of ecdysteroids appear, then, to contribute significantly to the observed increases in hormone titers during proecdysis; however, besides these long-term changes in the rate of elimination, the mechanisms regulating this elimination are capable of undergoing very rapid changes to bring about dramatic decreases in the endogenous hormone level. This rapid increase in the rate of elimination was observed in *G. lateralis* in late stage D_3. The rate coefficient for the elimination of 20-hydroxyecdysone increased from $k = 0.006$/hr to 0.05/hr at 24 to 36 hr before ecdysis to reduce the serum ecdysteroid titer from 100 to 15 ng/ml during the last day before ecdysis (McCarthy, in preparation).

An even more rapid change in the rate of elimination occurs following re-autotomy of partially regenerated limbs (LBA) in stage D_0. The rate of loss of 20-hydroxyecdysone from the whole crab increased ten-fold within hours of LBA (Figs. 2F, 3F). The rate of loss from the serum increased twenty-fold, while the loss from the carcass (serum-free) only doubled following LBA. This relationship suggests that ecdysteroids destined for excretion are drawn from the serum pool rather than directly from an intracellular compartment. The ecdysteroids that are rapidly lost just prior to ecdysis also appear to be drawn preferentially from the serum compartment (McCarthy, in preparation).

Although the increased rate of loss of radiolabeled 20-hydroxyecdysone from the serum following LBA ($k = 0.04$/hr) is sufficiently rapid to account for the observed decline in serum ecdysteroid titers ($k = 0.046$/hr; McCarthy and Skinner, 1977b), the nature of the regulatory mechanism(s) responsible for this enhanced rate of elimination have yet to be elucidated. Whatever the mechanism, it is clearly not regulated by nervous or neurosecretory factors originating in the eyestalks. Even when animals are deprived of eyestalks, the source of the postulated molt-inhibitory hormone, re-autotomy of partial regenerates in stage D_0 elicits the same responses as in crabs with intact eyestalks: serum ecdysteroid titers decrease very rapidly, proecdysis is temporarily suspended, and a secondary proecdysis is then initiated (McCarthy and Skinner, 1977b).

The stimulus for the increased rate of elimination may be a disruption of normal proprioceptive input. Re-autotomy severs the proprioceptive signals from the affected limb or limb regenerate; the rapid loss of ecdysteroid immediately prior to ecdysis may likewise be related to the detachment of the apodemes of muscles from the fragile exoskeleton during the preparation for ecdysis. Although there is no direct evidence for the role of proprioception on ecdysteroid metabolism, loss of proprioceptive input in larvae of the insect *Galleria* resulting either from sectioning the ventral nerve cord or from immobilization of larvae within glass tubes delays pupal apolysis. This inhibition is mediated by the subesophogeal ganglia apparently by direct neural connection to a target organ (Edwards, 1966; Alexander, 1970). A direct neural inhibition of the prothoracic gland was suggested, but the data are not inconsistent with an enhanced rate of elimination of the molting hormone.

Although many aspects concerning the nature of the regulatory mechanisms responsible for the interruption and re-initiation of proecdysis following limb autotomy in stage D_0 have yet to be resolved, the present study has: confirmed the rapid loss in serum ecdysteroids following LBA; demonstrated that this loss results from a rapid enhancement in the rate of catabolism and elimination of ecdysteroids from the crab; and demonstrated that whatever the unidentified mechanism regulating this response, its primary mode of operation is to rapidly

and directly affect the metabolism of the hormones responsible for regulating the molting process. These observations, as well as our pervious observations of the effect of eyestalk removal on serum ecdysteroid titers, limb regeneration and gastrolith deposition (McCarthy and Skinner, 1977a, b), make it clear that the regulation of the crustacean intermolt cycle is more complex and multi-faceted than suggested by the classic paradigm of antagonistic interactions between the eyestalk's molt-inhibitory hormone and the release of ecdysone from the Y-organ (Passano, 1960).

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Summary

1. The metabolic fate of injected [^{3}H]ecdysone was examined in crabs in premolt stage D_0 and in crabs at this stage whose proecdysis was interrupted by re-autotomy of partially regenerated limbs at the time of injection.

2. In both sets of crabs, 20-hydroxyecdysone was the principal metabolite, with further hydroxylation to 20,26-dihydroxyecdysone and the formation of polar conjugates (primarily in the midgut gland) occurring to a limited extent.

3. The rate of elimination of 20-hydroxyecdysone from normal crabs in stage D_0 is slow ($k = 0.002/hr$). Within a few hours after re-autotomy of partial regenerates from crabs in stage D_0, the rate of loss of 20-hydroxyecdysone from the whole crab increases by tenfold, while the loss from the serum accelerates twentyfold.

4. The increased rate of loss of 20-hydroxyecdysone from the re-autotomized crabs is associated with an increased amount of catabolism to polar inactivation products. The increase in the rate of elimination of ecdysteroids is sufficient to account for the observed decrease in endogenous ecdysteroid titers when proecdysis is interrupted by this re-autotomy.

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